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14. ABSTRACT
We continued to test MCF10A cells containing an inducible shRNA directed against the 3-UTR of BRCA2 for synthetic lethality with PARP inhibitors. Antibody staining with RAD51 and H2AX was used to look for double strand breaks and stalled replication forks and showed increase in RAD51 and H2AX positivity with knock-down of BRCA2 and PARP inhibitors. We also tried very hard to increase transfection efficiency in order to test wild-type and mutant forms of BRCA2 but were not able to transfect the large BRCA2 construct into these cells. We tried lipofection of various types, electroporation, and magnetofection. None of these methods were successful.

15. SUBJECT TERMS
Variants of Uncertain Significance, BRCA2, Synthetic Lethality, Parp inhibitors

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INTRODUCTION

An estimated ~22,000 women will be diagnosed with and ~14,000 women will die of **cancer of the ovary** in the United States in 2010[1]; approximately 10% of these cases will occur in heterozygotes for a hereditary breast and ovarian cancer syndrome (HBOC)[1] caused by deleterious *BRCA1* or *BRCA2* mutations[2]. Heterozygotes for *BRCA1* or *BRCA2* mutations have a 40% and 11-18% lifetime risk for ovarian cancer respectively, which is far greater than the 1.4% lifetime risk in the general population[3-5]. A pathogenic mutation in *BRCA1* or *BRCA2* is an important genetic biomarker for a high ovarian cancer risk in breast cancer patients and their unaffected female relatives; unfortunately 35% and 50% of all DNA variants detected in *BRCA1* and *BRCA2* respectively are variants whose pathogenicity is unknown and are termed "variants of unknown significance" (VUSs)[6]. These missense mutations of unknown significance are located throughout the entire coding sequence of the genes. **Without knowing if patients in whom a VUS is found really have a pathogenic *BRCA1/2* mutation, physicians do not know what to offer these patients and their relatives with regards to risk-reducing surgical procedures or surveillance.**

The functions of the *BRCA1* and *BRCA2* gene products are not completely known but they clearly operate in the homologous recombination (HR) pathway involved in double-strand break repair in DNA[7,8]. Poly [ADP-ribose] polymerase isoform 1 (Parp1) and its paralog Parp2 also have numerous functions in the cell but, in this context, it is the role of Parp1 in single-strand DNA break (SSB) repair that is probably most important[9-13]. It is estimated that 10^4 SSBs normally occur each day in a cell[14]. Pharmacological inhibition of Parp1 and Parp2, or siRNA knock-down of Parp1 alone[15,] causes a marked increase in SSBs. When a replication fork encounters such SSBs, they become DSBs, which can be detected as foci of phosphorylated γ-H2AX by immunostaining[16]. If not repaired, DSBs will cause collapse of the replication fork, leading to apoptosis. The cell normally avoids this catastrophic outcome by repairing the DSBs by homologous recombination, which requires the *BRCA1/2* gene products and can be visualized as foci of RAD51 by immunocytological staining[16]. This collaboration between Parp1 and *BRCA1/2* in DNA repair underlies the recently described synthetic lethal interaction (SLI) between them, a synergistic decrease in cell survival in cells with loss of function of either *BRCA1* or *BRCA2* and loss or inhibition of Parp1 activity[15,17]. SLI has generated intense interest as a new model for chemotherapy and a number of clinical trials of Parp inhibition for breast and ovarian cancers deficient in *BRCA1* or *BRCA2* are underway[18,19].

Hypothesis: Missense variants in *BRCA1* and *BRCA2* of unknown pathogenicity identified in cancer patients suspected of having an HBOC syndrome can be reclassified as being either functional (and therefore benign) or non-functional (and therefore pathogenic) based on their ability to complement the SLI caused by inhibiting Parp in cell lines deficient in *BRCA1* or *BRCA2* respectively.

Objective: Develop a robust assay by which individual VUSs can be tested for their effect on *BRCA1/2* function by expressing a *BRCA1* or *BRCA2* gene (cDNA) carrying the VUS into cell lines deficient in *BRCA1* or *BRCA2* respectively, exposing the cells to potent Parp

[1] Abbreviations: HBOC - Hereditary breast and ovarian cancer; *BRCA1/2* – Used when referring to both the *BRCA1* and *BRCA2* genes; VUS – Variant of unknown significance; BIC - Breast Information Core database; SLI -Synthetic lethal interaction: Parp - Poly [ADP-ribose] polymerase; HR – Homologous recombination; SSB – single-strand break in DNA; DSB – Double-strand break in DNA;ORF – Open reading frame; FACS- fluorescence activated cell sorting.

inhibitors, and determining whether the transfected gene is capable of protecting the cell from a SLI, leading to impaired cellular metabolism and loss of viability. **With better information in hand as to the functional significance of VUSs in *BRCA1* and *BRCA2*, patients and their physicians can assess ovarian cancer risk and make much better choices about surveillance and risk-reducing surgery and, ultimately, to more effectively prevent ovarian cancer in these potentially high risk patients.**

BODY OF REPORT

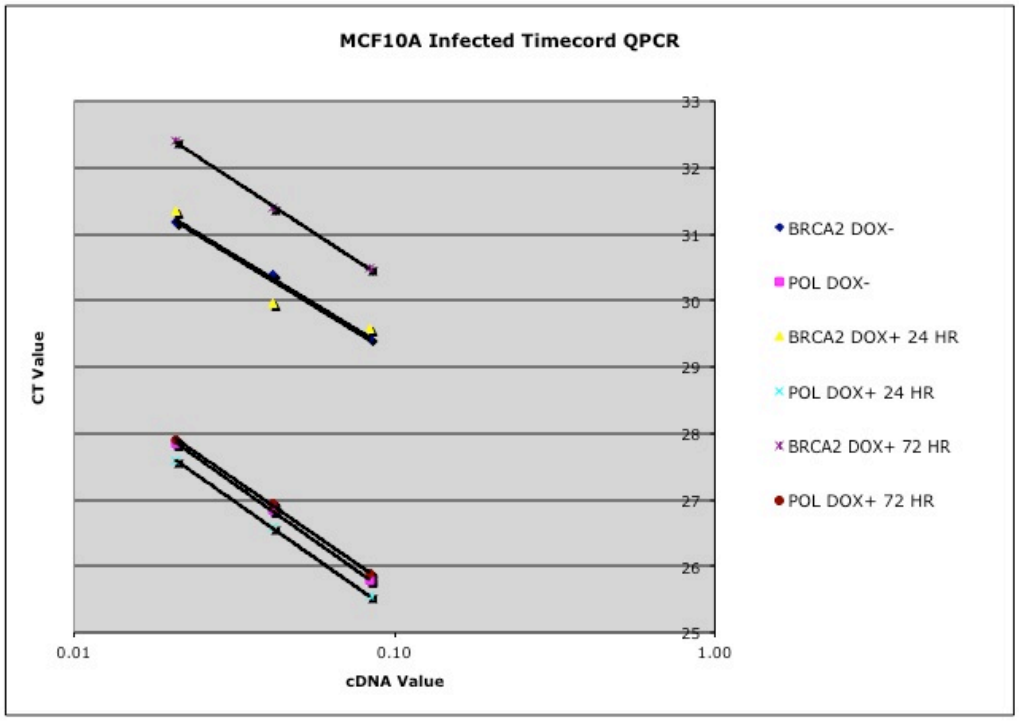
We have accomplished the following Aims of our grant proposal:

SOW Aim 1.

Creation of BRCA2 Knock-Down Cells using shRNA

As described in previous Progress Report, we found two shRNA constructs that in 293T cells were capable of knocking down BRCA2 expression by >70%. We selected one construct, #13 and used a lentivirus vector with a tet-inducible promoter to transfect the MCF10a cell line. Because the lentivirus also expressed an mCherry fluorescent protein, we used flow-sorting to pick the cells with the greatest fluorescence in order to have cell lines with the highest expression.

We used quantitative reverse-transcriptase PCR (qRT-PCR) to measure *BRCA2* RNA with and without doxycycline in the cells infected with shRNA lentivirus as an independent assessment of the effectiveness of knock-down.

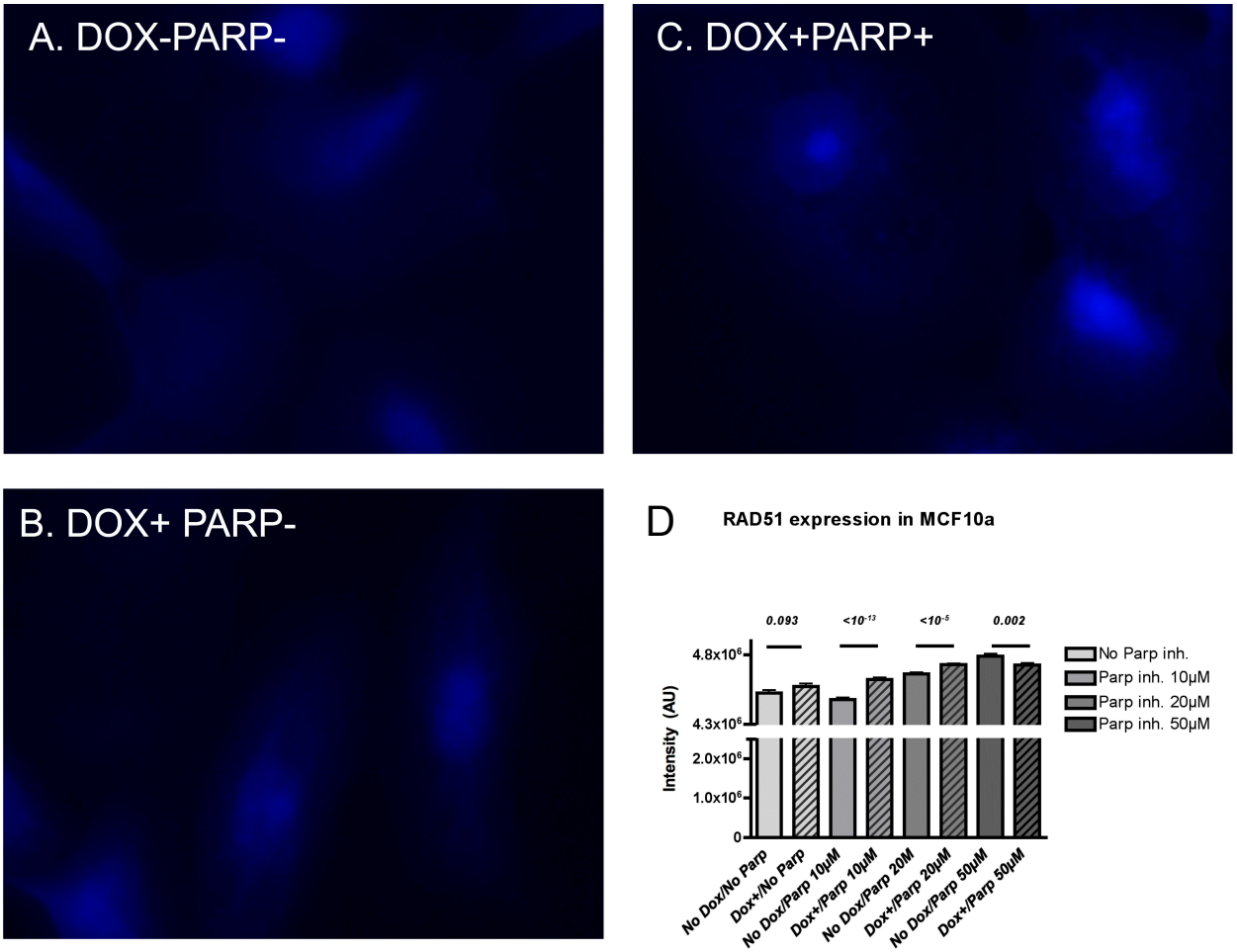


Shown here are the cycles for cDNA made from MCF10A cells containing the *BRCA2* shRNA, either without doxycycline or after 24 hours or 72 hours of 100 uM doxycycline. We used *BRCA2*-specific primers and, for reference, primers specific for DNA polymerase alpha. The cDNA was diluted 1/12 and then with two more two-fold dilutions (1/24 and 1/48). The Ct values are very linear, with r^2>0.999 for all but the BRCA2 qPCR after 24 hours because of a single point at the 1/24 dilution.

Using DNA polymerase alpha to calculate ΔΔCt for *BRCA*2 shows 24 hours of doxycycline caused a small reduction in BRCA2 mRNA, but 72 hours caused an increase in ΔΔCt of ~1, which translates to a 50% drop in mRNA. Recognizing that shRNAs work both by destabilizing mRNA as well as blocking translation, this degree of mRNA reduction is very consistent with a 70% reduction in protein seen by Western blotting.

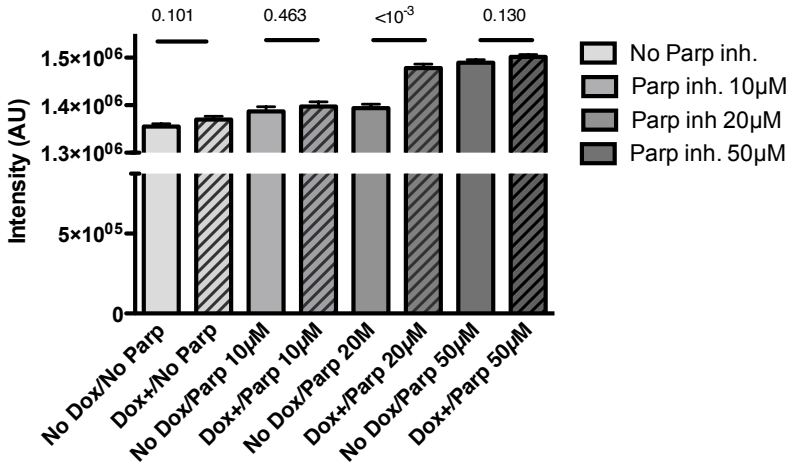
Synthetic Lethality between Poly (ADP-Ribose) Polymerase (PARP) Inhibition and Knock-Down of BRCA2

We had previously established a PARP inhibitor (PARPi) dosage of 10-20 micromolar as being effective in showing synthetic lethality as measured by colony forming assay. We then measured RAD51 and r-H2AX staining of nuclear foci to measure the degree to which doxycycline with or without PARPi increased double-strand breaks. Figure below shows increased intensity of staining when both doxycycline and PARPi are applied to MCF10a cells (Panel C) compared to when either knock-down alone (Panel B) or PARP inhibition alone (Panel A) is used. A summary of all of the experiments are shown in the accompany bar graph in panel D. There is a statistically significant trend of increased RAD51 intensity with both doxycycline and PARPi compared to either alone, although one set of data points, with 50 uM PARPi showed a small drop in the presence of doxycycline compared to without doxycycline.



A summary of staining intensity for RH2AX with or without BRCA2 knockdown and with or without PARP inhibition is shown below.

RH2AX expression in MCF10a



In the case of RH2AX expression, there is increasing intensity (measured in arbitrary units) when both a knock-down using doxycycline induction of the shRNA and PARP inhibition is present.

Transfection of BRCA2 into cells carrying inducible knock-down of BRCA2

We determined that magnetofection was the least toxic and damaging method of obtaining high frequency transformation of MCF10a cells with, for example, a Green-Fluorescent-Protein expressing vector. **Unfortunately, we could not say the same when we attempted to transfect full-length wildtype BRCA2 expression plasmid into MCF10a cells**. No matter what method of transfection we used, whether it was lipofection, electroporation, or magnetofection, we could not obtain sufficient numbers of transformed cells to be able to use a complementation assay for BRCA constructs carrying missense mutations of unknown functional consequences. We suspect there are two reasons for this: (1) The sheer size of the BRCA2 construct, 16.814 kb, is alreadyvery large and difficult to transfect; (2) overexpression of *BRCA2* at certain stages of the cell cycle is very toxic to cells (personal communication from Prof. Alan Ashworth). As a result, we cold not transfect many cells and, of those we did, without tight control of expression levels of the protein, we caused unacceptable toxicity insuccessfully transfected cells.

KEY RESEARCH ACCOMPLISHMENTS

- Development of two cell lines, a highly transformed 293T cell and a less-transformed epithelial cell line, MCF10A, each permanently infected with a lentivirus in which an shRNA against *BRCA2* has been inserted under control of a tetracycline inducible promoter. Treatment of these cells with doxycycline knocks down protein expression of *BRCA2* by >70% by Western blotting and *BRCA2* RNA by ~50% by qPCR.
- Demonstration that treatment of these cells with doxycycline induces cytological signs of synthetic lethality with Parp inhibitor by RAD51 and RH2AX focus formation.

REPORTABLE OUTCOMES

None

CONCLUSION

We have confirmed that we there is synthetic lethality for knock-down of BRCA2 and Parp inhibitors in breast epithelial cell line MCF10a, as determined by colony formation, and by RAD51 and RH2AX focus formation.

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